Silvio Aime
Dept.of Chemistry IFM & Molecular Imaging Center
University of Torino (Italy)

CONCEPTES OF PROBE DESIGN I: Physical Principles of Reporter moieties

Molecular Imaging [1,2] employs probes which are specific for a certain molecular event, which provides the basis for understanding of integrative biology, earlier detection and characterization of disease, and therapy evaluation, all in the same, living, but diseased subject. Molecular Imaging protocols should be performed in a relative rapid, reproducible, and quantitave manner, which enables the monitoring of time-dependent experimental, developmental, environmental, and therapeutic influences on gene and protein function in the same animal or patient. Such a multidisciplinary research uses the tools of molecular imaging which are based on existing imaging technology such as Positron Emission Tomography (PET), Single Photon Emission Computed Tomography (SPECT), Magnetic Resonance Imaging (MRI) and Optical Imaging (OI). Each of these imaging modalities has certain advantages and disadvantages, and the overall goal is to make use of and integrate the individual advantages of the single imaging modalities.
Imaging technologies for *in vivo* molecular imaging in small animals have undergone a fast development in the last five years, strating from a situation in which no dedicated equipment was available to a very intense competition about resolution and molecular sensitivity, both within one given technique and amongst different techniques. Most technologies used in the clinical field have now been adapted to the small animal resolution (μCT, μPET, μSPECT, μMRI). The following table reports the advantages and disadvantages of the most relevant imaging modalities [2].
The further goal in molecular imaging research is to combine the advantages of each imaging technology (e.g. high resolution for MRI, high sensitivity for PET, low costs and high sensitivity for Optical Imaging) into multimodal imaging to get the best possible answer to a certain scientific question.

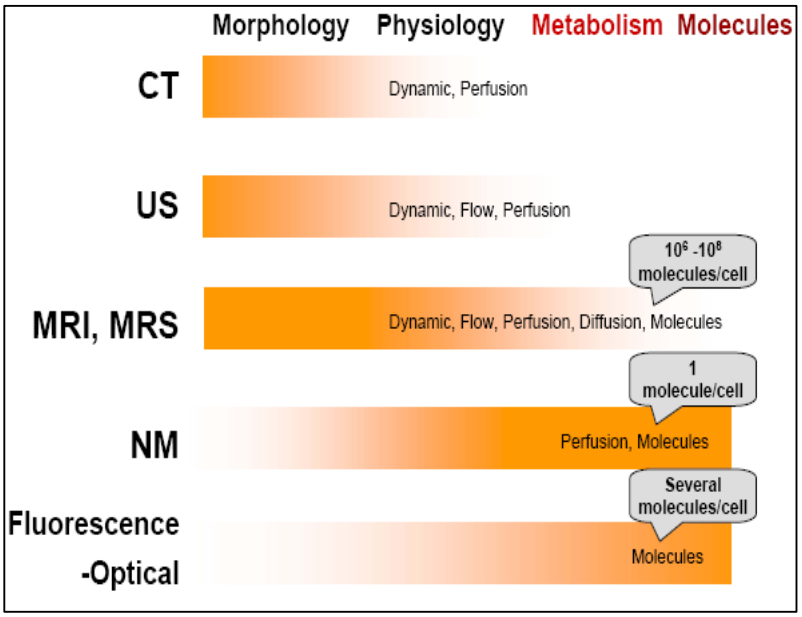

Table 1

Radionuclide techniques (PET, SPECT)

With regards to PET and SPECT, they both have benefited in terms of resolution from progress in crystal technology. Some improvement can still be expected for PET with the test of new crystals with better properties at 511 keV. However, the physical limits of PET tracer resolutions linked to the free path of β-electrons are almost reached, which will call for new breakthroughs to bring PET imaging at significantly sub-millimeter resolution [3]. With regards to SPECT, it is now clear that only alternative instrument architectures can bring SPECT to sub-millimeter resolutions.
The key-step in the widespread diffusion of PET imaging investigations is represented by the use of F-18 labelled FluoroDeossyGlucose (FDG) in the assessment of tumor metastases [4].

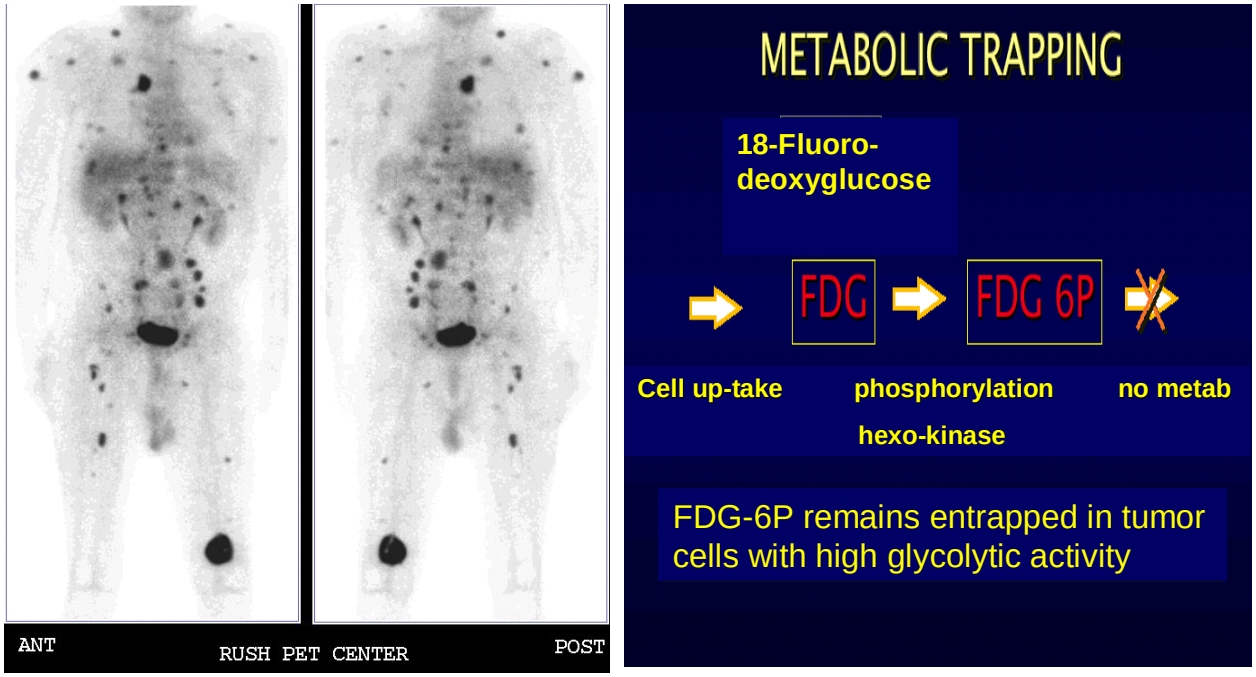

Fig. 1: ^{18}-FDG Application in PET Scan.

Receptor imaging for example, is an extremely powerful technique, and one that is also safe and flexible. However, both favourable bio-distribution and radio-nuclide characteristics are desirable. New chemistry based on the use of chelates of radioactive metal ions is expected to provide innovative solutions. Radioactive probes (for PET and SPECT) display an excellent sensitivity (nM), but the attainable resolution of the technique is rather poor (although an important development in "pre-clinical" nuclear medicine is the use of pinhole SPECT which displays an almost unlimited resolution, and small animal PET reaching local resolutions f about 1mm). Positron emitters like Zr-89, Cu-64 (produced in large amounts and high specific activity with "small biomedical cyclotrons") and Ga-68 (new Ge-68/Ga-68 generators available) present the radionuclidic basis for very promising radiopharmaceuticals. Can an analog of FDG be developed based on Ga-68, and a kit-type synthesis chemistry? That would be a breakthrough in PET Imaging to develop a "generator produced" positron emitter, very efficiently, at any PET center which does not have its own cyclotron! The availability of "generator produced" Ga-68 at low cost will

stimulate the development of chelator-coupled small molecules like peptides for different specificities. This may open a new generation of kit-formulated PET-radiopharmaceuticals similar to the ones in daily nuclear medicine practice based on the Mo-99/Tc-99m generator. Along with the long half-life of the generator, which can be used for more than a year, Ga-68-based radiopharmaceuticals may become a very cost-effective alternative to cyclotron-based tracers. Supposed the rather short physical half-life of Ga-68 of 68 min is not adequate to the molecular imaging of longer-lasting biological processes, a similar PET radionuclide generator might be developed: Ti-44/Sc-44 ($T_{½}$ = 4.8 h).
SPECT tracers are mainly represented by Tc-99m and In-111 containing species. Tc-99m is the most widely used radionuclide in diagnostic medicine to image different organs and a wide range of different disease states (e.g. cardiac and kidney function and the spread of cancer to bone). Tc-99m ($t_{1/2}$ = 6.02h, Eγ = 141KeV) possesses ideal properties for medical radio-imaging because the γ-emission is sufficiently energetic to allow for the visualization of sites deep within the human body by scintillation cameras without exposing patients to high level of ionizing radiation. The half-life time of 6 h provides sufficient time for labelling, administration and biodistribution without having to use excessive amounts of radioactivity to compensate for losses due to radioactive decay. An important invention that made Tc-99m widely available to all hospitals is the Mo-99/Tc-99m generator, which has been developed at the Brookhaven National Laboratory in the late 1950s. Mo-99/Tc-99m generators are based on the decay of the parent Mo-99 to Tc-99m wich occurs with 87.5% efficiency. The useful time of a generator is typically of 1-2 weeks.

Optical Probes

Imaging technologies based on optics have appeared only recently and have been so far restricted to animal research. Nevertheless, it is expected that some of them will be in the shorter term adaptable to clinical use. Fluorescent Imaging (FLI) and Bioluminescent Imaging (BLI) can be succesfully used in studying specific cell- and tissue-promoter, to follow trafficking and fate of cells expressing GFP or Luciferase, to assess apoptosis, protein-protein interaction and gene-transfer. Due to their high sensitivity, FLI and BLI are extremely useful for molecular imaging applications, including cell labelling. Microscopy optical imaging by confocal and multiphoton microscopy possess impressive sensitivity and spatial resolution, though they suffer of the limited penetration of light through biological tissue. However improvements can be foreseen with the use of time resolved fluorescence based approaches.

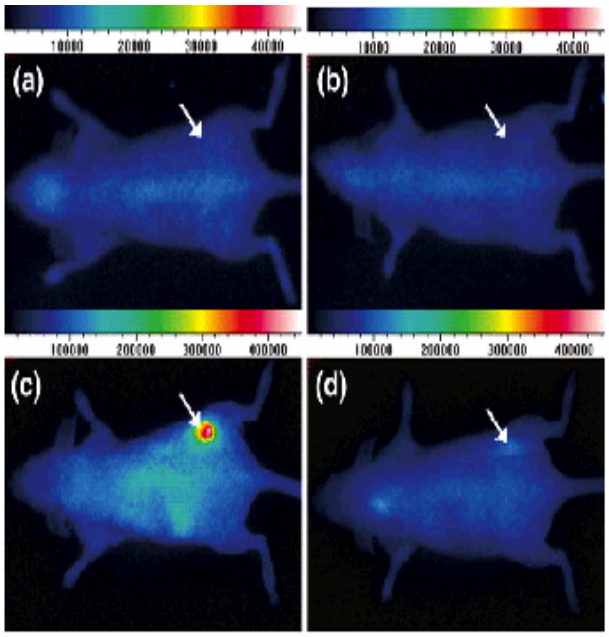

Fig. 2: Tumor bearing mice (RIN38 pancreatic tumor expressing somatostain receptor) treated with a somatostatin analog ocreotate coupled with indocarbocyanine dyes (0.02 μmol/kg iv). Enhanced tumor is visible at 6h after injection

MR-Imaging Probes

The superb spatial resolution and the outstanding capacity of differentiating soft tissues have determined the widespread success of MRI in clinical diagnosis [5,6]. The main determinants of the contrast in a MR image are the proton relaxation time T1 and T2. It is now well established that when there is a poor contrast between healthy and pathological regions due to a too small variation in the relaxation times, the use of a contrast enhancing agent can be highly beneficial. Contrast agents are chemicals able to alter markedly the relaxation times of water protons in the tissues where they distribute. According whether the dominant effect occurs mainly on T1 or T2, MRI contrast agents may be classified as positive or negative agents, respectively. The most representative class of T1-positive agents is represented by paramagnetic Gd(III) chelates whereas iron-oxide particles represent the class of T2-negative agents.

Iron Oxide particles

These are water insoluble systems that yield very strong T2 effects as the result of dramatic long-range disturbance in the magnetic field homogeneity. These agents are made of a crystalline core of superparamagnetic Fe(III) oxide (SPIO, maghemite, γ-Fe_2O_3) surrounded by coating materials like dextran or carboxydextran. The diameter of the iron-oxide core is just 3-5 nm whereas the overall particle may be of 50-200 nm diameter. Two products are available for clinical use: Endorem® (Guerbet) and Resovist® (Schering). These agents provide excellent (negative) contrast when administered at doses as low as 8-15μmol/kg body weight. Once administered intravenously, as particles, these agents accumulate in the cells of the

reticuloendothelial system. The phamacodynamic properties of the iron oxide particles are affected by both the size and the overall electric charge. The smaller particles remain in the blood circuit for a time long enough to be considered as blood pool agents for angiographic assays.
For molecular imaging applications the external cover is functionalized with the proper vector.

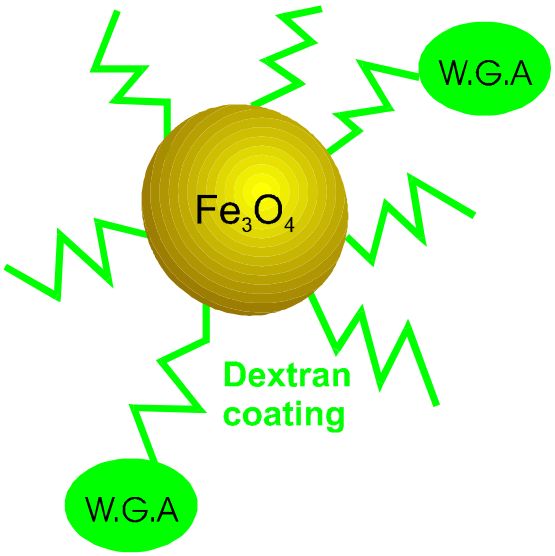

Fig. 3

A typical example of functionalization is represented by the conjugation of USPIO particles to transferrin (TF). Being TF receptors overexpressed in tumor cells, the USPIO-TF probe has been successfully used to visualize tumor cells in a rat-mammmary carcinoma model [7].

Gd-complexes

The currently used Gd(III)-based contrast agents distribute in the vascular and extravascular space and are particularly useful to report about lesions in the central nervous system. A very active area of research deals with the dynamic use of contrast agents, since the kinetics of their distribution in the extravascular space is related to the vascular permeability. For instance, newly formed vessels functional to the tumor growth display permeability much higher than normal capillaries, an issue that s tackled also by assessing the changes in the expression of vascular endothelial growth facttor (VEGF) receptors. Thus, research in the field of dynamic contrast enhancement has a strong synergism with thr development of molecular imaging procedures aimed at reporting a direct visualization of the expression of VEGF receptors. Much of the current knowledge on how improving the relaxivity of Gd(III) chelates relies on the work done for the development of angiographic agents. One succesful approach consists of designing functionalized Gd(III)chelates displaying high binding affinity to human serum albumin (HSA) [8]. Upon formation of the macromolecular adduct between the Gd(III) chelate and HSA, a marked relaxation enhancement takes place, that is primarly related to the increase of the molecular reorientational time (τ_R) □n going from the free to the bound form. An additional contribution to the observed relaxivity arises from exchangeable protons and water molecules on the surface of the protein in close proximity to

the binding site of the paramagnetic agent. The theory of paramagnetic relaxation foresees relaxivities upto 100 mM-1s-1 and more (calculated at 20MHz) for complexes containing one water molecule (q=1) bound to HSA. However, it has been often observed a "quenching" effect on the attainable relaxivity due to a long exchange life-time(τ_M) of the coordinated water molecule. Therefore, efforts have been made to get a better understanding of the determinants of the exchange rate of a water molecule coordinated to a Lanthanide(III) ion.

Actually, promising routes to high relaxivities may be envisaged by the use of Gd(III) chelates containing two or even three water molecules in the inner coordination sphere of the metal ion. As far as the delivery of large number of Gd(III)-complexes at a targeting site is concerned, several systems are currently under intense scrutiny, including dendrimers, liposomes, and other forms of lipophilic aggregates. The overall size attainable with these systems may limit their accessibility to tagets located on the endothelial wall.

A nice example of targeting on endothelial site has been reported by Sipkins et al. [9] in the targeting of a specific angiogenesis marker, the endothelial integrin $\alpha_v\beta_3$, whose presence has been shown to correlate with tumor grade. The imaging probe used in this work is a Gd(III)-based polymerized liposome. This approach provided enhanced and detailed detection of carcinoma through the imaging of the angiogenic vasculature.

Recently, the same $\alpha_v\beta_3$ target has been addressed with lipidic nanoparticles containing a huge number of Gd-chelate units (94400 Gd/particle). One of the lipidic component is covalently bound to the $\alpha_v\beta_3$-integrin peptidomimetic antagonist. Further routes have been explored to target receptors in solid tissues. For instance, Bhujwalla and coworkers [10] have recently developed and applied two-component Gd-based avidin biotin system for the visualization of HER-2/scan receptors, a member of the epidermal growth factor family over-expressed in multiple cancers. Their approach consisted of addressing the extracellular domain of the receptors by means of a biotinylated monoclonal antibody (mAb). After clearance of the unbound mAb, Gd-labeled avidin is administered and binds, with high affinity, to the biotinylated mAb. The method has been succesfully applied in an experimental mouse model breast carcinoma.

Another example based on recognition properties of the biotin/avidin pair has been reported by Kobayashi et al [11] who investigated the uptake of a macromolecular construct comprised of avidin and a biotinylated dendrimer bearing 254 Gd-DTPA chelates into SHIN3 cells (a cell line obtained originally from human ovarian cancer). The internalization process is driven by the recognition of a β-D-galactose receptors, present in some cancer cells, especially ovarian and colorectal adenocarcinoma cells by avidin.

An interesting route for amplifying the MR signal has been developed by R. Weissleder and coworkers [12]. Their approach is based on the enzyme-mediated polymerization of paramagnetic substrates into oligomers of higher relaxivity. They used Gd-chelates functionalized with phenolic substituents which undergo rapid condensation in in the presence of H_2O_2 and peroxidase. The increased molecular size of the oligomeric structures causes an increase of the molecular reorientational time which, in turn, results in an increase of the observed relaxivity. This approach has been applied to the imaging of E-selectin-peroxidase conjugate.

Often, after targeting vector has delivered the imaging probe to the proper site at the membrane surface, an internalization process may take place that leads to the intracelluar entrapment of the imaging probe.

Several routes leading to the cell internalization of Gd-based systems have been explored, namely pinocytosis, phagocytosis, via membrane receptors (including receptor-mediated endocytosis) and transmembrane transportes, by the use of membrane translocation peptides and byelectroporation.

The contrastographic eficacy of the cell-internalized MR-imaging probe may depend upon its intracellular distribution. Recently [13], in order to get more insight into this issue, the relaxation enhancements measured for a MR-image probe (Gd-HPDO3A) cell-internalized by pinocytosis or by electroporation have been compared. Whereas the former route causes the entrapment of the contrast agent into endosomic vesicles, the latter one leads to its disperion into the cytoplasm.

Electroportaion consists of the formation of transient hydrophylic pores on the cell membrane upon application of suitable electric pulses between the two electrodes placed in the cell suspension. As shown in figure, the relaxation rate of the cells labeled by pinocytosis shows a saturation effect upon increasing the amount of the internalized Gd-HPDO3A with a limiting R_1 value of ca 3 s^{-1}.

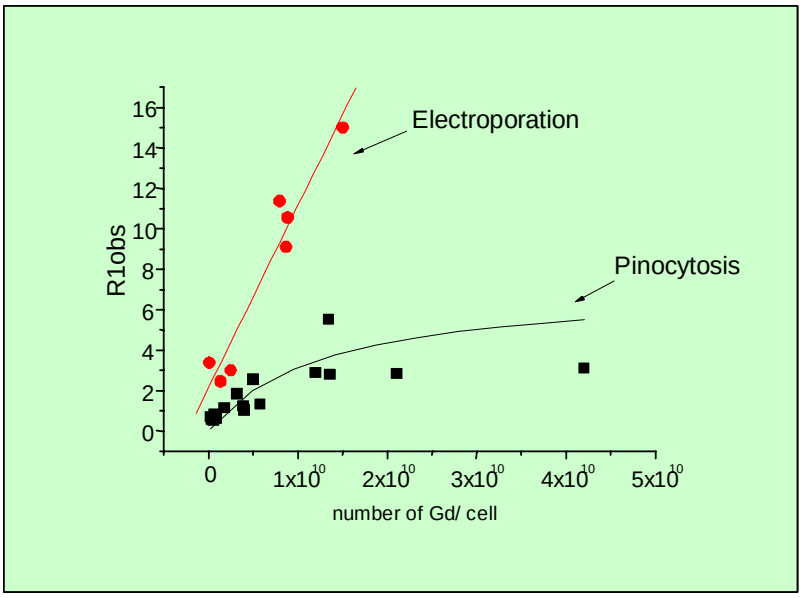

Fig. 4

Conversely, the R1 values for the cellular pellets labelled by electroporation are markedly higher, and even more importantly, they are linearly dependent upon the amount of the internalised complex. A cellular pellet (and a portion of tissue as well) can be considered as a multi-site system where the water molecules are distributed in the extra- and in the intra-cellular (or cytosolic) compartments. Such compartments are separated by the cellular membrane, whose water permeability is crucial for determining the relaxometric behaviour of the whole pellet. The behaviour observed in Fig. 4 may be explained in terms of a three-site water exchange model when the imaging probe is entrapped into endosomes (extracellular/cytoplasm/endosome compartments) and in terms of a two site exchange model when the paramagnetic agent is only dispersed into the cytoplasm. On this basis, the "quenching" effect of the exchange on the relaxation rate of cytosolic water protons is the responsible factor for the saturation of the relaxation rate observed at high concentrations of the internalized probe for the HTC pellets labelled by pinocytosis. The obtained results remark the importance of the procedure used for labelling cells and demonstrate that the cytosol confinement of the probe yields higher relaxing efficiency, in turn allowing the MRI detection of a smaller number of cells with respect to the intrapment into endosomes.

CEST agents (Chemical Exchange Saturation Transfer)

CEST agents formally belong to the class of negative contrast agents because they determine a reduction of the signal intensity of water protons in MR images. Differently from other negative agents (e.g. iron-oxide particles) their effect is not due to the T2-shortening, but to a saturation transfer mediated by chemical exchange.
The fundamental requisite for a molecule in oder to act as CEST agent is the presence of a set of mobile protons (resonating at a frequency ω^{CEST}), whose exchange rate with water protons (resonating at ω^{WAT}) must be smaller than the $|\omega^{CEST} - \omega^{WAT}|$ difference. When this condition is

met, the application of a proper radiofrequency pulse, centered at a frequency ω^{CEST}, will cause the saturation of the mobile protons of the CEST agent. This saturated magnetization will be, then, transferred to the water protons by chemical exchange, thus resulting in a decrease of the signal intensity of the latter. A peculiarity makes CEST agents unique in the scenario of MRI contrast agents: the contrast is generated only if a specific irradiation frequency, characteristic of the given CEST agent, is applied. As a direct consequence, more than one set of mobile protons may be irradiated provided that they are encoded with sufficiently different resonance frequencies. This property is extremely advantageous, because it makes possible the set-up of ratiometric methods where the contrast is made independent on the absolute concentration of the CEST agent.
The well known remarkable effect on the chemical shift values induced by the presence of a paramagnetic enter can be exploited for the design of paramagnetic CEST agents, recently termed PARACEST agents, in which the exchangeable protons are part of the complexes containing the paramagnetic metal ion. The main advantage of such systems, mainly represented by lanthanide chelates (Ln ≠ Gd), relies on the large increase of the $|\omega^{CEST} - \omega^{WAT}|$ difference that makes possible the irradiation of fast-exchanging protons (e.g. water protons directly coordinated to the paramagnetic center), thus leading to very efficient CEST agents [14]. Furthermore, for PARACEST agents endowed with more than one set of mobile protons, the spread-out of the resonance ferquencies induced by the paramagnetic center makes easier the selective saturation required by the ratiometric method . Currently, one of the main limitations of the CEST agents is represented by their relatively low sensitivity, but interesting developments are currently under intense scrutiny.

References:

1) Massoud TF, Gambhir SS, Molecular imaging in living subjects: seeing fundamental biological processes in a new light, GENES & DEVELOPMENT 17 (5): 545-580 MAR 1 2003
2) Rudin M, Weissleder R, Molecular imaging in drug discovery and development, NATURE REVIEWS DRUG DISCOVERY 2 (2): 123-131 FEB 2003
3) Phelps ME, Positron Emission Tomography provides molecular imaging of biological processess, Proc. Nat. Acad. Sci. USA, 97, 9226-9233, 2000.
4) Oyajobi BO et al, Imaging tumor burden by [F-18]FDG-PET and osteoblast activity by [Tc-99m]MDP-SPECT/CT in the 5TGM1 mouse model of myeloma bone disease., JOURNAL OF BONE AND MINERAL RESEARCH 20 (9): S212-S212 Suppl. 1, SEP 2005
5) I.R. Young, Methods in Biomedical Magnetic Resonance Imaging and Spectroscopy; John Wiley & Sons: Chichester (2000).
6) P.A. Rinck, Magnetic Resonance in Medicine; ABW Wissenschaftsverlag GmbH: Berlin (2003).
7) Kresse M., Wagner S., Pfefferer D., Lawaczeck R., Elste V., Semmler W. *Magn. Res. Med.* 40, 236, 1998
8) Aime, S.; Botta, M.; Fasano, M.et al. E. In Merbach, A.E.; Toth, E. Editors. The chemistry of contrast agents in medical Magnetic Resonance Imaging. Chichester, John Wiley & Sons, LTD, 2001 p. 193.
9) D.A. Sipkins,D.A. Cheresh, M.R. Kazemi, L.M. Nevin, M.D. Bednarski, K.C.P. Li Nat. Med. 4 (1998) 623.
10) Z.M. Bhujwalla , D. Artemov, N. Mori, R. Ravi, Cancer Res. 63 (2003) 2723.

11) Kobayashi H, Kawamoto S, Saga T, et al. Bioconjugate Chem 2001; 12, 587-593.

12) Querol M, Chen JW, Weissleder R, Bogdanov A, ORGANIC LETTERS 7 (9): 1719-1722 APR 28 2005

13) Terreno E, Geninatti Crich S, Belfiore S et al. Magn. Med. Res. 2006, 55, 491-497.

14) Aime S, Carrera C, Castelli DD, Crich SG, Terreno E, ANGEWANDTE CHEMIE-INTERNATIONAL EDITION 44 (12): 1813-1815 2005